(*NEW SERIES.*)

No. 26.

SCIENTIFIC MEMOIRS

BY

OFFICERS OF THE MEDICAL AND SANITARY DEPARTMENTS

OF THE

GOVERNMENT OF INDIA.

LEUCOCYTOZOON CANIS.

BY

CAPTAIN S. R. CHRISTOPHERS, M.B., I.M.S.

ISSUED UNDER THE AUTHORITY OF THE GOVERNMENT OF INDIA
BY THE SANITARY COMMISSIONER WITH THE GOVERNMENT
OF INDIA, SIMLA.

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LEUCOCYTOZOON CANIS.

SEVERAL examples of parasites resembling the hæmogregarine forms so common in the cold-blooded vertebrates have recently been found in the blood of mammals. One of these parasites occurring in the white blood corpuscles of the dog, is the subject of the present memoir. As seen in the peripheral blood, it has already been described by James (1) and by Bentley (2); but neither of these authors has investigated the parasite in the organs, and since this parasite of the dog was the first of the series to be described, neither was in a position to compare it with other mammalian hæmogregarines. These omissions, as far as possible, have been made good in the present memoir which, describing among other matters the free motile vermicule, and the formation in the red marrow of reproductive cysts, adds many new facts to what was previously known regarding the parasite.

James, who found the parasite in six out of 45 dogs, examined by him in various parts of Assam, has given a very complete description of it as seen in the peripheral blood. He does not state whether the dogs were pariah dogs or dogs of European extraction, though the context strongly suggests that they were adult dogs of the latter kind. He describes the parasite as occurring in the polymorphonuclear leucocytes, and as consisting of a very characteristic oblong, nucleated, unpigmented body contained in a definite capsule or cytocyst, formed from the body substance of the leucocyte in which the parasite is situated. He notes the difficulty with which the parasite stains, drawing attention to the fact that the parasite itself is often not seen, the area occupied be its capsule then appearing as a clear space. In well and deeply stained specimens he was able to display the parasite, which lay, as a rule loosely, in its oval cytocyst.

James observes that the leucocytes are but little affected by the presence of the parasite and that their amœboid movement is not interfered with.

Besides the common forms, James mentions others of rarer occurrence, which are more circular in shape, and in which the contour of the parasite is only very indistinctly marked off from the general body protoplasm of the leucocyte. These forms he conjectures may be a less mature stage of the ordinary encapsuled parasite.

He describes in the parasite a round or oval nucleus, which is generally situated close to one extremity. Bodies containing an elongated chromatin mass centrally situated are also noted, and the nucleus, according to James, frequently seems to be made up of a number of small round chromatin-granules.

The escape of a parasite from a cell was observed once by James. After eaving the cell it was worm-like, more pointed at one end than the other. It showed slow vermicular movements, but did not travel from the spot at which it was liberated James concluded that the parasite had affinities with the hæmogregarines and represented an entirely new form of mammalian blood infection. He thought it should be recognised as the type of a new genus and gave to it the name Leucocytozoon Canis.

According to James, Bentley, though his account is of later date, was the first actually to see the parasite. It is interesting to note, with reference to what comes later, that Bentley describes parasites as found also in transitional and mononuclear leucocytes.

Shortly after the discovery of this hæmogregarine-like parasite in the dog, similar parasites were described by Balfour (3) and myself (4) as occurring in tne Gerboa (Jaculus gordoni), and in the Indian field rat (Gerbillus indicus), respectively. The former has been named by Laveran (5), *Hæmogregarina balfouri*, and the latter I have called *H. gerbilli*. Both these forms occur in the red blood corpuscles of their host and do not differ, in any recognisable way, from the hæmogregarines of cold-blooded animals. In the red cell they are partly bent upon themselves so as to form a 'tail,' and like hæmogregarines they have an active vermicule stage in which they are free in the plasma.

Patton (6) shortly after described another hæmogregarine *H. funambuli* found by him in the Kathiawar squirrel (*F. pennantii*), and Balfour (3) one almost identical in appearance in the rat (M. decumanus). Both these parasites invade the leucocytes but differ from *L. canis* in choosing mononuclear elements. They lie in spaces in the cell but have no definite capsule. The chromatin mass of L. funambuli, and apparently also of the rat parasite, stains with the same ease as the nuclei of the leucocytes. A characteristic hæmogregarine-like 'tail' is often present in *L. funambuli*, and free vermicules occur.

Another distinct species described by Patton (1) is *L. felis domestici*. It occurs in the polymorphonuclear leucocytes of bazaar cats about Madras. It is more closely related to *L. canis* than to the other species. It lacks, however, the capsule of *L. canis* and resembles the naked oval form of this species mentioned later more than the encapsuled one.

Leucocytozoon canis, which had hitherto been reported only from Assam, I have found to be quite common in the pariah dog about Madras. Though very common in pariah puppies it is rare in the adult dogs. Added to the fact that James seems to have found the parasite in adult European dogs, this recalls the conditions relative to European and indigenous child malaria.

It is difficult to say exactly what percentage of the puppies of Madras are infected. One reason is that extremely scanty infections are rather common

and prolonged search often results in the finding of a few or even a single parasite in the blood of a dog which the result of a less thorough examination would have led one to suppose was free from infection. Again, neglected dogs are especially liable to harbour the parasite, whereas well conditioned puppies are much less frequently infected. The figures one arrives at, it is obvious, will depend very much on the proportion of dogs belonging to the former or latter class respectively which one deals with. In the case of neglected puppies picked up from the bazaar, and taking into consideration scanty infections, the number infected is nearly 100 per cent. and at least 25 per cent. of such dogs show heavy infections. Material being so readily obtained, it was thought desirable to supplement such work as had already been done on this parasite by some further research.

No special methods were employed. Nochts Romanowski, Giemsa's stain and Novy's modification of Romanowski's stain have all at times been used. The last stains the forms more intensely than either of the former, but requires to be used with great care since it has so great a tendency to deposit. Giemsa's stain readily colours the ordinary encapsuled parasite, but it often will not touch the young reproductive cysts. The action of Nocht's Romanowski stain depends entirely upon the particular brew of stain that is used. Some brews give the most beautiful results, other quite fail to stain the encapsuled parasite in spite of the fact that they colour the nuclei of the leucocytes deeply.

For the detection of developmental forms in the bone marrow, smears stained with Giemsa's or Nocht's stains are searched with a $\frac{1}{8}$-inch dry objective. This enables one to pick out the large mature cysts, and even to detect the small unstained refractile forms.

The mature cysts are beautifully displayed in fresh squash preparations of the bone marrow.

Vermicules are difficult to obtain except from the gut of ticks (*R. Sanguineus*) fed on infected dogs.

They are studied quite readily in the gut, especially in that of the nymph. In the case of nymphs removed from heavily infected dogs, the number of vermicules is very great. Fresh preparations are best made by adding a little of the gut contents to a drop of normal saline and, after mixing the two, covering. Dry smears are best made by dragging a portion of the gut over the slide, or by breaking up a piece of the gut in normal saline, before spreading with the needle. Smears made by spreading the undiluted blood from the gut are apt to be unsatisfactory, as blood in the tick is generally of a tarry consistence.

It would be superfluous after James' very thorough account to describe again the characters of the parasite as it is seen in the peripheral blood. All the mammalian hæmogregarines are totally unlike any other form of blood parasite

found in mammals and the appearance of the dog parasite is especially striking. This is especially the case in specimens stained in a routine manner with Romanowski's stain for, owing to the refusal of the parasite to stain it is often seen while all else in the specimen is perhaps deeply coloured, only as beautifully regular oval brilliantly transparent clear spaces in the leucocytes, or as bodies faintly tinged with blue lying in this situation in their clear cyst-like capsules.

The resistance of *L. canis* to staining is undoubtedly due to the capsule. The capsule, according to James, is formed by a condensation of the protoplasm of the leucocyte about the parasite. In my specimens this appearance of condensation is very marked, the parasite being surrounded by a line of more deeply staining protoplasm, which in some specimens appears to consist of closely aggregated granules. This line on its outer edge merges imperceptibly into the surrounding protoplasm of the leucocyte, but, on its inner edge, ceases so abruptly against a clear space as to suggest that the parasite, in addition to a layer of altered protoplasm, is surroun ded by a capsule proper to itself. Several consid erations support such a view. Not only is the edge of the oval space very defined, but the contour is extremely regular, and the capsule, though described as oval, is sufficiently flattened at the poles to give the impression of living form, rather than merely of a space distended with fluid. It is significant, too, that the contained parasite, however bulky, never quite touches the condensed protoplasm of the leucocyte, but leaves between itself and this a clear even line.

In stained films one not infrequently encounters forms quite free in the plasma, and these possess the clear oval capsule, though they are unsupported by any apparent cell substance.

The appearances in fresh preparations, especially when a trace of methyl violet is present, are particularly suggestive of a true capsule, for the encapsuled parasite appears as a solid and even rigid foreign body, often piercing the leucocyte from side to side.

Perhaps the strongest reason of all for believing that the capsule of *L. canis* is a true cyst, is that in the leucocyte parasite of the squirrel, though a cytocyst is present, none of the above appearances are seen. There are also forms of *L. canis* to be described later, which, though surrounded by a layer of condensed protoplasm, stain readily.

The hæmogregarines of the red cells, *H. gerbilli* and *H. balfouri*, merely lie in a space in the red cell and though in *H. gerbilli* the red cell may, in its altered state, stain so that it resembles a thin enveloping membrane, there is no trace of any proper capsule.

Within the capsule, in properly stained specimens, is seen the quadrangular, bean-shaped, or vermicule-like parasite. The ordinary forms, so far as I have

been able to ascertain, are without any trace of the 'tail' so characteristic of *H. gerbilli*. The chromatin mass, which lies close to one end of the parasite, stretches almost if not quite across the body of the parasite which is here at its narrowest.

The chromatin mass is like that of *H. gerbilli* composed mainly of strands, which pass transversely to the body of the parasite forming a rather open coarse reticulum. The protoplasm is finely reticular and near the thicker extremity of the parasite is often vacuolated. Owing to the position of the chromatin mass at one end of the parasite the protoplasm is as a rule almost all collected in front of this body. But there are forms in which the chromatin mass is more centrally situated and the protoplasm more evenly divided.

The protoplasm may or may not exhibit granules. The number and appearance of the granules varies with the stain employed and the depth of staining achieved. When weak Nocht's Romanowskis stain is used, the protoplasm is blue and reticular, devoid in most cases of any granules. In specimens coloured with Giemsa's stain fine reddish speckling is generally visible, and a few small more distinct granules are often present. Novy's modification of Romanowski's stain brings out numerous and quite large granules.

The invaded cells are said by James to be polymorphonuclear leucocytes, and, after the examination of some of James' preparations which he was kind enough to send me, I was myself convinced that it was this kind of cell that was invaded. But further work on the subject and especially comparison of cells containing *L. canis* with those invaded by *L. funambuli* has led me to modify my opinion. It is some time since I saw these preparations of James', and I have unfortunately not been able to re-study them, but considering the matter from my new point of view, I have been struck by the fact that, though James depicts in fig. 5 of his plate an invaded cell in which the nucleus differs from that of a polymorphonuclear leucocyte only in being more opened out, all his other figures, with one doubtful exception, represent cells the nuclei of which are not obviously polymorphonuclear in nature.

As a matter of fact the great majority of cells in which parasites occur do not conform exactly to any normal type of leucocyte. The nucleus stains more diffusely and often more intensely, than the nuclei of other leucocytes. Most commonly two masses of nuclear matter of which one is usually larger than the other, are seen lying on each side of the parasite (fig. 2, a, d). Such a nucleus is apt to give rise to different appearances which depend a good deal upon the amount of flattening the cell has undergone in the making of the film. Very often the nucleus is split up into a number of separate pieces, a condition which seems to have resulted from the parasite pushing its way through and through this structure, since every stage between slight indentation of the nucleus by the

parasite and the condition where the nucleus is completely broken up, can be made out. This fragmented condition of the nucleus is almost exactly reproduced in mononuclear leucocytes of the squirrel which have been invaded by *L. funambuli*. The protoplasm of invaded cells, though it may contain a few fine granules resembling those in the polymorphonuclear cells, is usually pale and devoid of granules, and the outline of the cells is indistinct and often difficult to follow in its entirety.

In fresh films invaded cells exhibit amœboid movement, but in most cases it is quite clear that the cells are crippled by the presence of the parasite, for their movements lack the vigour of those of healthy leucocytes, and in the time that a normal cell will have traversed several fields of the microscope, they will scarcely have moved from their original position.

As previously stated, these cells are unlike any normal type of leucocyte; even after much work I was at first unable, by direct observation, to be quite sure what normal element the invaded cell did actually represent. This uncertainty was increased by the fact that cells containing *L. funambuli* were often identical in appearance with those invaded by *L. canis*. It was only as a result of observations on the bone marrow that a satisfactory solution of this question was found. As will be seen later, the cell in the peripheral blood is a cell which invaded in its mononuclear stage in the bone marrow, has developed towards a transitional stage, and possibly even may have reached a polymorphonuclear stage, though modified by the destructive influence of the parasite acting throughout its whole life period.

The existence of a motile vermicule stage in *L. canis* is suggested by the single observation of James. This stage I have since been able to study both in fresh and in stained films. In fresh preparations of the blood free vermicule forms are seldom seen, *L. canis* in this respect offering a marked contrast to *H. gerbilli* and to *L. funambuli* in both of which vermicules are easily found in ordinary fresh blood preparations.

Though rare *in vitro*, the vermicules of *L. canis* occur in large numbers and are readily studied in the gut of dog ticks (*R. Sanguineus*) which have fed on infected dogs. Seen in the gut of the tick, the vermicules exhibit active movements. Very characteristic is a movement which consists in a gradual flexion of the body, thus approximating the two extremities of the parasite followed by a rather sudden movement of extension. The majority of vermicules have one extremity much more pointed than the other, but stouter, more sausage-shaped forms are also occasionally seen, exhibiting a characteristic rotatory movement, which may be transmitted to masses of blood corpuscles or other debris. In stained films the vermicules show a sharp and a blunt extremity. The sharp end is transparent and free from granules; the blunt

end is more granular and stains more darkly. In the centre of the parasite is an oval chromatin mass, which stains intensely. As a rule the chromatin mass is more compact than it is in the encapsuled forms, but vermicules with a chromatin mass identical in appearance with that of the encapsuled parasite are quite frequently seen. Even in stained films the tendency to lateral flexion is shown by the curved position which the vermicules have adopted. (Fig. 3*a*.)

Encapsuled forms are very rarely found in the tick, and the vermicules are so abundant as to suggest that almost all the parasites here take the form of active vermicules.

The function of the vermicules of the hæmogregarinidae is not yet altogether clear. Hintze (8) has shown that in Lankesterella vermicules may perform sexual functions. In *H. clamatae* (Stebbins) the parasite uses a free motile stage to pass from cell to cell apparently for nutritive purposes only. The liberation of vermicules on the slide such as takes place in *H. gerbilli*, though it suggests some extra corporeal function, may denote only the escape of trophozoite forms under the unusual stimulus of removal from the tissues. The conditions relating to the formation of vermicules in *L. canis* are in this connection very significant. As will be seen later, parasites which invade cells are those in a stage of development having the chromatin composed of separate rod-like granules, while parasites which have lain in cells long enough to become encapsuled have a chromatin mass of quite a different character. Since forms seen invading cells in the bone marrow never have chromatin like that of the encapsuled forms, it is clear that these latter do not leave their host cells, in which they have become encapsuled, to invade others. The behaviour of the encapsuled parasite, under the stimulus of the conditions in the gut of the tick, is therefore extremely suggestive of its leaving its capsule normally only in relation to extra corporeal development.

In the bone marrow, forms are seen resembling those in the peripheral blood, but there are in addition, other forms which are rarely seen elsewhere, and others, again, which are confined to this tissue.

Almost as numerous as the encapsuled forms, are parasites entirely different in appearance. These are round, or egg-shaped bodies, which stain readily and have no capsule. (Pl. figs. 4 and 5.) They are about the same size, but never so elongate, as the encapsuled forms, the average measurement being 9μ across in the circular forms and 12μ by 8μ in the egg-shaped forms. The protoplasm in my specimens is most often of the same tint as that of the cell in which it lies, but it is at times paler or, more rarely, darker. There is usually to be made out, between the protoplasm of the parasite and that of the cell, a fine interval. Outside this there is generally a condensation of the protoplasm of the leucocyte, such as occurs in the encapsuled forms, but this is sometimes

absent or little marked, in which case the presence of the chromatin mass alone attracts one's attention to the parasite.

The chromatin mass is very distinctive, and quite unlike that of the encapsuled form. It consists of small isolated rod-shaped granules, thirty or forty in number, arranged loosely in a circle, somewhat as is the pigment in a malarial crescent. When not free, these forms are invariably found in one kind of cell only. These cells are of mononuclear type and possess characters which will be described later.

These round and oval naked forms abound in the bone marrow, but also occur in much smaller numbers in the liver and spleen; in the peripheral blood they are rare. It is undoubtedly this variety of the parasite which James has noted as being occasionally seen in the peripheral blood. From considerations that will appear later, it will be seen that he was correct in thinking this to be a less mature stage of the parasite.

Though, between the encapsuled parasite with reticular chromatin mass and the naked form with scattered chromatin granules, there is little apparent connection, yet it is not difficult to find varieties which exhibit every gradation between the one and the other. Thus some parasites, though they are surrounded by an oval space, stain readily and exhibit the scattered arrangement of the chromatin, and others resembling ordinary encapsuled forms so far as their capsule is concerned, can still be seen to be in a transition stage since their chromatin consists of a number of aggregated but separate granules. That one form of parasite changes into the other, and that this change consists in the development of the naked form into the encapsuled form seems quite certain. Any doubt of this sequence is dispelled by a study of the changes which go on in the invaded cell.

I have already briefly touched upon the question of the kind of cell in which the parasites occur, but since the invasion by a parasite of the leucocytes whilst these are still in their immature stages in the marrow, is an entirely undescribed condition, it is important, because of the possible bearing of the matter on the origin of leucocythæmic conditions in man, to ascertain exactly, not only the behaviour of the parasite, but such cell re-actions as it may give rise to in its host.

The examination of films of peripheral blood of dogs coloured with Romanowski's stains enables certain types of cells to be clearly differentiated.

(*a*) Cells in which the nucleus is narrow and lobulated, showing a coarse reticular structure, and the protoplasm of which, when intensely stained, exhibits numerous fine red granules. Such cells normally form about forty per cent. of the total number of leucocytes. They are exactly comparable with the polymorphonuclear leucocytes of man, and resemble these in every particular.

In preparations stained with weak Nocht's Romanowski stain, the fine granules are often not visible, and the protoplasm shows up clear and transparent with a pinkish tinge, contrasting with that of the second type of cell to be described, where, under similar conditions, the protoplasm is of a purple colour, owing to the presence of indistinct granules which have taken up the basic stain. (Fig. 12.)

(*b*) Cells in which the nucleus may be mononuclear, quadrangular or horse-shoe-shape, in which the protoplasm when intensely stained exhibits a certain number of fine red granules together with much bluish granular matter. In specimens coloured with weak Romanowski's stain the purple or even blue granular protoplasm contrasts, on the one hand with the clear pink or colourless protoplasm of the polymorphonuclear cells, and on the other with the hyaline blue protoplasm of those cells which correspond with what, in human blood, one is accustomed to call large and small mononuclear leucocytes. Though many of these cells, especially those having horse-shoe-shaped nuclei, approximate in appearance to the polymorphonuclear cells, they yet form a type so easily distinguishable as to merit their inclusion under a separate type-head. Such cells as we have described correspond to the transitional cells of human blood. In the dog they form about 20 per cent. of the total leucocytes. (Fig. 13.)

(*c*) Cells of mononuclear type of large size having hyaline basophyllic protoplasm. Though these cells most resemble the large mononuclear leucocytes of man, they differ from these in possessing more regularly oval nuclei and protoplasm which stains more intensely. (Fig. 15.)

(*d*) Cells of intermediate or small size with round nuclei and hyaline somewhat basophyllic protoplasm. They may have comparatively voluminous protoplasm, or a thin layer only around the nucleus. They often contain a few large and distinct red granules. Cells of this type are practically identical with the small mononuclear leucocytes of man. Neither in this nor the last mentioned type of cell do parasites occur. (Fig. 15.)

(*e*) Cells with irregular shaped nucleus the protoplasm of which is crowded with large eosinophyl granules. Though in a few particulars they differ from the eosinophyl cells of man they undoubtedly correspond to these. They are not as a rule numerous, but they may constitute as much as 18 per cent. of the total leucocytes. They are never invaded by parasites. (Fig. 16.)

(*e*) Mast cells. These resemble the mast cells of man. They are only very occasionally seen.

In the red marrow, though the great variety of cell forms is at first rather confusing, it is possible to distinguish with certainty the following:—

(*a*) Cells with large circular or oval nuclei showing fine reticular structure the protoplasm of which stains more or less markedly blue. These are the neutrophyl myelocytes. Stained with hæmatin and eosin, their nucleus is pale

and faintly indicated. I have never been able to detect parasites in this type of cell. (Fig. 18c.)

(*b*) Cells with round deeply staining nuclei having a very coarse reticular structure, the protoplasm of which is intensely basophyllic (figs. 18 and 19). These cells are peculiar on account of the depth of stain they exhibit. Next to the myelocytes and type (*c*), they are the most numerous cells in the marrow. They are not invaded by parasites. (Fig. 18*d*.)

(*c*) Cells usually possessing a circular outline and clear or finely reticular protoplasm. The nucleus is mononuclear, quadrangular, dumb-bell shaped, or in the form of a horse-shoe. They resemble the transitional cells of the peripheral blood, though examples of this type with mononuclear nuclei are much more numerous in the marrow than in the blood. This type is undoubtedly the polymorphonuclear cell of the blood in its early stages of development. For convenience of description I shall term this type of cell transitional. It is all important in the present connection, since it is cells of this type which are selected by the parasite for its host. (Fig. 18*a* and 18*b*.)

When discussing the relation between the naked and encapsuled form, I showed that the former was the early stage of the latter. The same relation holds good in the case of the cell in which these two forms occur. The cells in which the naked forms occur beyond a slight indentation of the nucleus by the parasite, exhibit no degenerative changes. They are mononuclear forms of transitional cells, or more rarely cells of this type having dumb-bell-shaped nuclei. By the time the parasite has become encapsuled, this cell, which would normally have progressed through various transitional stages to that of a mature polymorphonuclear leucocyte, has by reason of its own development and the action of the parasite come to possess the fragmented, more or less diffusely staining nucleus, and transparent protoplasm of the cell we have already described. The accompanying table, which records in a graphic form parasites encountered in the examination of the marrow of an infected dog, and the variety of cell they occupied, shows this sequence very clearly :—

TABLE I.

Circular or oval naked forms with scattered chromatin.	m	m	m	m	m	f	m	m	m	m	m	m						
Parasites intermediate between encapsuled and naked forms.	m	h	m	m	m	m	h											
Ordinary encapsuled forms . . .	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a

Parasites encountered are signalled by a letter signifying the type of cell occupied—
m = Mononuclear transitional cell.
h = Transitional cell with horse-shoe-shaped nucleus.
a = Altered cell.
f = Free form.

Though in my specimens, the parasite has practically always lain in altered transitional cells, yet it is quite conceivable that the factor of normal development of the cell being greater, or the effect of the parasite less, a condition would result where the including cell was still recognisable as a polymorphonuclear. This appears to have occurred more frequently in James' than in my own case, a fact probably due to differences in the age or condition of the dogs with which we have each had occasion to deal.

It is difficult to believe that the condition of infection described can exist in any degree without modifying the number and proportion of the leucocytes in the blood, and since up to the present no parasite attacking immature leucocytes in the marrow has been known, the nature of any such change could not be predicted.

Patton has noted in the case of *L. funambuli* that in severe infections the number of mononuclear leucocytes is increased, the proportion of these reaching in some cases 80 per cent., whereas in the normal squirrel (*F. palmarum*) they form only about 30 per cent. of the total leucocytes.

James in his memoir gives differential leucocyte counts of four infected dogs. The number of polymorphonuclear leucocytes in these cases varied from 58·2 per cent. to 67 per cent., a proportion which does not suggest any very great increase of their numbers.

In the following table a series of blood counts of healthy and infected dogs are given. From this it will be seen that the total leucocytes of uninfected dogs vary between 9,000 and 18,000 per c.m.m. The polymorphonuclear cells form from 30 per cent. to 64 per cent. and the transitional cells from 11 per cent. to 26 per cent:—

	Dog 1. Puppy not infected.	Dog 2. Puppy not infected.	Dog 3. Puppy not infected.	Dog 4. Puppy not infected.	Dog 5. Puppy Proplasma abundant.	Dog 6. Adult not infected.	Dog 7. Puppy not infected.	Dog 8. Adult Proplasma scanty.	Dog 9. Puppy Hæmogregarines abundant.	Dog 10. Adult Hæmogregarines scanty.	Dog 11. Puppy Hæmogregarines abundant.	Dog 12. Puppy Hæmogregarines abundant.	Dog 13. Puppy Hæmogregarines abundant.	Dog 14. Puppy Hæmogregarines abundant.
Polymorphonuclear	59	64	56	53	46	53	56	31	66	42	53	67	61	54
Transitional	20	21	24	15	26	15	17	11	23	22	15	12	21	1
Large mononuclear	4	6	3	3	6	3	3	4	3	3	4	4	3	1
Small mononuclear	14	9	17	11	7	11	16	39	5	25	19	14	11	28
Small mononuclear with granules.	3				20			8	35					
Eosinophyl						18	2	7	5	4	9	3	4	2
Total leucocytes per c. m. m.				18,000	9,700		12,700	12,000		6,015	11,400	12,800	23,000	
Total red cells per c. m. m.				4,475,000	1,200,000		3,912,500	4,950,000		5,450,000			3,100,000	

The percentage of mononuclear cells in my dogs has been very low, possibly a result of poor nutrition. In some instances infected dogs have exhibited marked leucocytosis, but parasites are often abundant when no such change is to be observed, and, since leucocytosis is rather common in native dogs, one must be careful in drawing conclusions as to its significance.

Any very marked alteration in the proportion of leucocytes is absent and though there is, in severe infections, usually some increase in the number of the polymorphonuclear and transitional cells, yet there is nothing at all suggestive of the profound changes which occur in leucocythæmia.

Turning from the discussion of the influence of the parasite on the tissues, we have still to consider the means by which it reproduces itself in the body of its host.

In 1898 Laveran (10) described in the cells of the liver reproduction forms of *H. stepanovi* a hæmogregarine of the tortoise. This parasite, in the liver cells, forms cysts containing from 12 to 20 sporozoits. Since then, several hæmogregarines of cold-blooded animals have been found to reproduce themselves by encystment in the cells of the viscera, and the formation of sporozoits. Similar cysts have lately been described by Balfour (3), and Laveran (11), in the mammalian form *H. balfouri*. The cysts of *H. balfouri* are circular or oval and reach 21μ to 23μ in diameter. According to Laveran they usually contain 16 sporozoits. But Balfour figures cysts containing over 30 sporozoits. Balfour states that the cyst wall is formed only by the cell substance of the liver cell, but Laveran notes that, though not present in other hæmogregarines, there seems in this case to be a true cyst membrane.

The encysted stage of *L. canis* I have found in the bone marrow. It is very doubtful if it occurs in either the liver or the spleen, for in dogs in which cysts were quite common in the bone marrow, they were not found in these organs. Mature cysts are oval or circular, measuring up to 48μ in diameter. They possess a wellmarked and thick hyaline cyst wall, around which are the flattened remains of cells. Within the cyst lie at least 30 sporozoits. These are sausage shape and contain a central rather irregular dark staining chromatin mass. (Fig. 11.)

Less mature cysts are correspondingly smaller. They are exceedingly resistant to stain, and are filled with round or oval clear refractile granules of large size. As the cyst matures, the granules become less in number and sporozoits are seen lying among them. Later on the refractile granules all disappear, and the cyst contains only sporozoits and a little granular matter. Still younger stages are brilliantly clear and refractile. They are oval in shape and lie in an altered cell. The cysts appear to arise from ordinary encapsuled forms, which become swollen so that they are more broadly oval. Such forms are extremely

difficult to stain. The contained parasite, when stained, is seen as a short vermicule lying against one side of the capsule. The chromatin mass is situated in the centre of the vermicule, and is much broader than in the ordinary forms. It is not uncommon to find parasites, whose capsules are of normal shape, lying against one wall of the capsule and having the chromatin mass in the centre. Such forms are narrow and the chromatin mass is elongated. It is possible that these forms are about to become encysted. (Fig. 9*a*.)

The method of transmission from dog to dog I have been unable to ascertain. Several of the hæmogregarines of cold-blooded vertebrates have been suspected by different observers to be carried by blood-sucking annulata. Durham (11) states that the carrier of a species of *drepandium*, discovered by him in the toad, is a tick. He describes, in the tick, conjugation and the formation of large cysts. Siegel (12) believes *H. stepanovi* to be carried by a leach (*placobdella*) and describes in the gut of this blood-sucker the formation of gametocytes, fertilisation and further development. The carrier of *Karyolysus lacertarum*, according to Schaudinn, is the tick *Ixodes ricinus*. Laveran and Negre (13) describe in *Hyalomma Aëgyptium* a protozoon parasite, which they think may be the extra corporeal form of a hæmogregarine.

In the case of hæmogregarines of mammals, I have described in a former publication (14) the development of *H. gerbilli* in the louse [*Hæmatopinus stephensi* (*Newstead and Christophersi*)] which infests *gerbillus indicus*. Vermicules swarm in the gut and large cysts are found in the body cavity of lice fed on infected field rats.

I have already noted that liberation of the vermicules of *L. canis* takes place in the gut of the tick (*R. Sanguineus*) both in the nymph and in the adult stage, but I have been unable to trace any further development. The dissection of adult ticks which have hatched from nymphs fed on heavily infected dogs has always yielded negative results, and not even vermicules in such cases have been recovered from the gut.

Other possible carriers than the tick have to be considered. In addition to ticks, all my dogs have been infested with fleas (*P. serraticeps*). A species of *menopon* is extremely common. This insect, which resembles a louse in its habits, is extremely apt to be conveyed from animal to animal, and often swarms upon sickly dogs, or dogs kept in close confinement. The species, though a false louse, feeds upon blood. A constant ecto-parasite of neglected dogs is a species of spider-fly (*hippobosca*), peculiar to the dog, which frequents especially the inside of the ear, and the folds of the groin and the bite of which frequently causes large pustular vescicles. Mosquitoes also feed freely on dogs. If *L. canis* be transmitted by a blood-sucker, it is probably by one of the above mentioned, since dogs, found by repeated

examination to be free from infection, have in many cases become infected after being some weeks in the laboratory where, so far as one could see, only the above species of blood-suckers were present.

The zoological position of *L. canis*, in so far that it comes within the division hæmogregarina as defined by Laveran, is quite clear. Its exact position in this group requires further discussion. Of mammalian hæmogregarines six have been so far described, namely, *L. canis* (*James*), *H. balfouri* (*Laveran*), *H. gerbilli* (*Christophers*), *L. funambuli* (*Patton*), *L. felis domestici* (*Patton*) and the parasite of the Norway rat (*Balfour*). Of these, I have been able to study side by side all but the parasite of the Norway rat.

Of these forms *H. gerbilli* is so exactly similar to many cold-blooded hæmogregarines that I have no hesitation whatever in placing it in the genus Hæmogregarina (*sensu restricto*) of Danilewski. *H. balfouri* is very closely related to *H. gerbilli*, and this species, too, I place without hesitation in the same genus. The difficulties of classification and nomenclature arise only when one approaches the forms which are found in leucocytes.

L. canis is a very curious parasite, differing from the other species more especially in the possession of a very thick and distinct capsule, but also in that the chromatin mass is extremely close to one end, whilst, so far as I have been able to make out, no semblance of a tail is ever present. *L. funambuli* does not resemble *L. canis* so much as it does *H. gerbilli* or *H. balfouri* and the parasite of the Norway rat from Balfour's description seems to be indistinguishable from *L. funambuli*. The similarity between this species and *H. balfouri* is shown by the fact that Balfour has considered them as possibly the same species in different hosts.

The position of *L. funambuli* in the genus *leucocytozoon* depends at present upon the fact that it attacks the leucocytes, not to my mind in itself a sufficient reason. The retention of James' genus leucocytozoon for the dog parasite has the additional support that morphologically the parasite is unlike the other forms. Whether these morphological differences are sufficiently great to make the foundation of a new genus desirable is doubtful.

A point not to be lost sight of is that, *L. funambuli* in particular, and *L. canis* and *L. felis* to a less extent attack the nucleus, pushing their way through this in a very distinct manner. In *L. funambuli* it is often evident that the parasite has again and again traversed this structure. This behaviour raises the question whether these forms may not be related to *Karyolysus* (*Labbe*), a conjecture which has to support it the fact that *L. canis* and *L. felis* possesses the other character required by Labbe's definition of the genus that it is shorter than its host cell. Even *L. funambuli* and the parasite of the Norway rat would quite easily take their position in this genus.

Until more information is forthcoming regarding the hæmogregarines of mammals it is perhaps as well for the present simply to retain the genus leucocytozoon, placing in this genus *L. canis*, *L. felis*, *L. funambuli* and the parasite of the Norway rat, *H. gerbilli* and *H. balfouri* being considered as representing mammalian forms of Hæmogregarina (Danilewski).

To summarise—*Leucocytozoon canis* comes within the division *Hæmogregarina* of Laveran and very possibly represents the mammalian form of *Karyolysus (Labbe)*. Reproduction occurs by the formation of true cysts, containing about 30 sporozoits. Encystment takes place in cells of the bone marrow. After escaping from the cysts, the sporozoits invade mononuclear transitional cells in the marrow, where they are seen as naked oval forms. These undergo changes and become encapsuled, whilst the host cell is altered in a characteristic way.

Though the parasite is essentially a parasite of the bone marrow and of cells which give rise to leucocytes, no very great changes in the leucocyte value are produced.

In the gut of the dog tick (*R. Sanguineus*) and more rarely in fresh preparations of blood, parasites escape from their capsules as active vermicules.

Possible carriers are the dog tick, the dog flea, a species of *menopon*, the mosquito and *hippobosca*.

NOTE.

Since writing the above account of *Leucocytozoon canis* (James), I have been able to follow the complete sexual development of this parasite in the tick (*R. Sanguineus*, Latreille).

In twenty-four hours after the tick has dropped from its host many of the vermicules have lodged themselves in the large cells of the gut, especially the younger cells near the basement membrane, and are associated in pairs. On the second day conjugation between two similar vermicules takes place. As a result of conjugation a globular body is formed containing a single large and very homogenous mass of chromatin. This, after conjugation is complete, spreads outwards to form a more and more extended reticulum and the protoplasm, which has begun to take a deep blue stain, increases rapidly in amount. The chromatin eventually occupies the periphery of the parasite and collects into irregularly arranged star-shaped masses. The whole body by the third or fourth day splits up to form from eleven to fourteen sporozoits. The sporozoits resemble the original vermicules but are thinner. The chromatin mass also is more globular and more compact. When set free the sporozoits are in the lumen of the gut, the parasite having been carried gradually inwards by the growth of the cell in which it has embedded itself.

Development can be followed in the nymph as far as conjugation and the formation of the young zygote, but by the fourth or fifth day every sign of the parasite has disappeared. No development could be traced in the larva or in the adult male. Examination of the ova so far has also yielded negative results, so that the method of re-entry into the dog still requires elucidation.

Ticks fed on uninfected dogs do not shew vermicules or the other stages described.

Our knowledge o the life cycle of *Leucocytozoon canis* is therefore very nearly, if not quite, complete. Reproduction in the bone marrow of the mammalian host must be looked upon as schizogony, whereas sporogony takes place in the gut of the tick.

The detailed description of the process of sporogony will form the subject of another memoir.

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EXPLANATION OF THE FIGURES IN THE PLATE.

Fig. 1 *a*. A parasite in a leucocyte. Fresh preparation.
b. Vermicules. Fresh preparation.
Fig. 2 *a*. A leucocyte containing a parasite which has refused to stain. Note the edges of the nucleus overlapping the capsule.
b. A leucocyte containing a parasite which has stained. Note the layer of condensed protoplasm around the capsule.
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d. A leucocyte containing a parasite which has stained faintly. Note the bridge of nuclear matter lying over the capsule.
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a. Form in fixed position.
b. Very common posture of vermicules of *L. canis*.
c. Showing sharp anterior extremity.
Fig. 4. Naked form.
Fig. 5. Naked form surrounded by a layer of condensed protoplasm of the leucocyte
Fig. 6. Parasites showing commencement of capsule formation.
Fig. 7. More advanced stage of above.
Fig. 8. Unusual form of parasite.
Fig. 9. *a*. Long form of parasite with centrally situated elongated chromatin mass. (?) First stage in encystment.
b. Commencing cyst formation.
c. Later stage in encystment.
Fig. 10. Nearly mature cyst in the bone marrow.
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Fig. 12. Polymorphonuclear leucocyte.
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Fig. 15. Mononuclear leucocytes with basophyllic hyaline protoplasm.
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Fig. 17. Parasite which has attacked one limb only of the nucleus of a transitional cell.
Fig. 18. Red marrow.
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b. Transitional cell with horse-shoe-shaped nucleus.
c. Myelocyte.
d. Cell of type *b*.
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b. Encapsuled parasite lying in an altered transitional cell.
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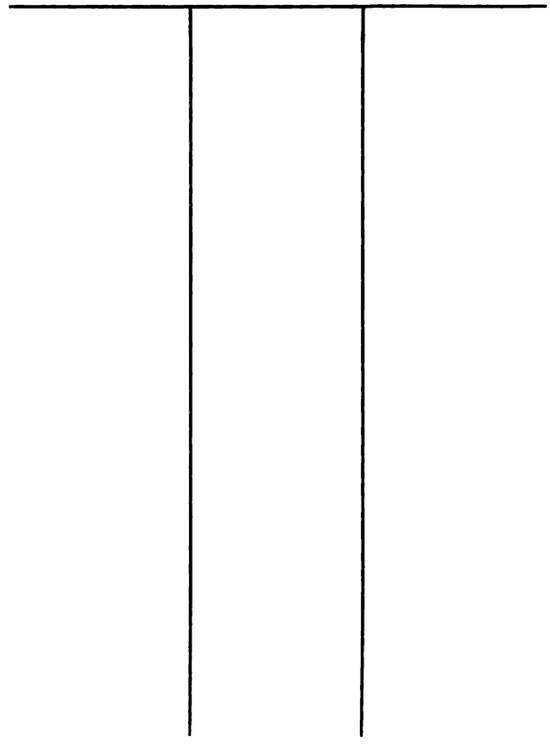

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